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AMPA-induced Ca^{2+} influx in cultured rat cortical nonpyramidal neurones: pharmacological characterization using fura-2 microfluorimetry

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Abstract

Immunocytochemical and Co^{2+} uptake studies revealed that in primary cultures of rat cortical neurones, the majority of neurones are γ-aminobutyric acid (GABA) immunopositive and can express Ca^{2+}-permeable α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) receptors. By fura-2 microfluorimetry, it was shown that the stimulation with the selective agonist (*S*)-AMPA (0.3–300 μM) induced a concentration-dependent but cell-variable increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (EC_{50} value 7.4 μM) in more than 80% of the medium-sized multipolar neurones studied. The AMPA-induced rise in $[Ca^{2+}]_i$ seems to be due to Ca^{2+} entry through AMPA receptor channels, because the response was abolished in Ca^{2+}-free solution and by AMPA receptor selective antagonists, but was not significantly influenced by cyclopiazonic acid, an inhibitor of the endoplasmatic Ca^{2+}-ATPase, by selective *N*-methyl-D-aspartic acid (NMDA) receptor antagonists, as well as the Na^+ channel blocker tetrodotoxin and the majority of tested Ca^{2+} channel blockers. In conclusion, the results indicate that the cerebral cortical neurones in culture represent mostly GABAergic interneurone-like cells and the majority of them possess Ca^{2+}-permeable AMPA receptors, important for intracellular signal transduction and neuronal plasticity.

Keywords: AMPA receptor; Cortical neurone, cultured; GABA (γ-aminobutyric acid) immunocytochemistry; Co^{2+} uptake; Fura-2 microfluorimetry; Ca^{2+} concentration, intracellular

1. Introduction

The three ionotropic glutamate receptor classes, α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA), kainate (non-NMDA) and *N*-methyl-D-aspartic acid (NMDA) receptors, play fundamental roles in excitatory neurotransmission and, probably, in the aetiology of several brain diseases (for review, see Michaelis, 1998; Ozawa et al., 1998; Dingledine et al., 1999). AMPA receptors, which mediate fast excitatory synaptic transmission in the mammalian brain, are thought to be tetra- or pentameric complexes assembled from four different protein subunits (termed glutamate receptor GluR1-4 or GluRA-D subunits) (Hollmann and Heinemann, 1994; Yamada, 1998). Additional structural diversity is generated by mRNA editing and alternative splicing of the flip/flop module (Burnashev and Rozov, 2000).

The possibility that Ca^{2+} ions may permeate through glutamate receptor channels has stimulated considerable interest. It is now well accepted that Ca^{2+} plays an important role in mediating various (patho)physiological consequences of glutamate receptor activation, like plastic changes in synaptic transmission, long-term potentiation or excitotoxic neuronal cell death (Michaelis, 1998; Ozawa et al., 1998). Unlike NMDA receptors, the majority of AMPA/kainate receptors are usually associated with minimal permeability to Ca^{2+} (Mayer and Westbrook, 1987; Hollmann and Heinemann, 1994; Burnashev, 1996). However, several investigations have demonstrated the existence of native AMPA receptors with increased Ca^{2+} permeability in distinct cell populations, e.g., hippocampal, cortical and amygdala interneurones (Iino et al., 1990; Jonas et al., 1994; Geiger et al., 1995; Mahanty and Sah, 1998), cerebellar neurones (Brorson et al., 1999) or spinal motoneurones (Vandenberghe et al., 2000). These receptor channels can directly mediate significant Ca^{2+} influx and are important for the increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$) (Iino et al., 1990; Geiger et al., 1995). It is remarkable that in principal neurones of neocortex and hippocampus, AMPA receptors

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show a low Ca^{2+} permeability, whereas in nonpyramidal cells, the Ca^{2+} influx was markedly higher (Jonas et al., 1994; Jonas and Burnashev, 1995). The relative permeability to Ca^{2+} depends strongly on the edited GluR2 subunit in that its presence inhibits Ca^{2+} influx (Sommer et al., 1991; Hollmann and Heinemann, 1994). However, considering the recently reported molecular and functional diversity of AMPA receptors, e.g. in hippocampal interneurones (Angulo et al., 1997; Washburn et al., 1997), further studies with selective pharmacological tools seem to be of special interest.

In line with previous microfluorimetric investigations to characterize NMDA receptors in cultured mesencephalic and cortical neurones in our laboratory (Allgaier et al., 1999; Scheibler et al., 1999), the aim of the present study was to analyze the AMPA receptor-mediated Ca^{2+} influx in cultured rat cortical nonpyramidal neurones. We especially investigated to which extent these neurones, which seem to have a better chance to survive than pyramidal cells under culture conditions, express AMPA receptors with high Ca^{2+} permeability. Some of the data have been communicated in abstract form (Fischer et al., 1999, 2000).

2. Materials and methods

2.1. Materials

The following drugs and chemicals were used: Dulbecco's modified Eagle medium/Nutrient mixture F12 media, Hank's balanced salt solution, trypsin, gentamycin (GibcoBRL, Life Technologies, Paisley, UK); mouse monoclonal anti-GABA (γ-aminobutyric acid) antibody (ICN Biomedicals, Aurora, OH, USA); 1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-4,5-dihydro-3-methylcarbamoyl-2,3-benzodiazepine (GYKI 53655; gift of Dr. L. Harsing, Institute for Drug Research, Budapest, Hungary); foetal heat-inactivated bovine serum (Seromed, Biochrom, Berlin, Germany); DNase (Roche, Mannheim, Germany); (*S*)-α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), *R*(−)-2-amino-5-phosphonopentanoic acid (R-AP5), calcicludine (CaC), *R*(−)-3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP), 6-cyano-7-nitroquinoxaline-2,3-dione disodium (CNQX), cyclopiazonic acid, cyclothiazide, cytosine-β-D-arabinofuranoside, 3,3′ -diaminobenzidine tetrahydrochloride (DAB), diazoxide, dimethyl sulfoxide (DMSO), EGTA, flunarizine dihydrochloride, fura-2 acetoxymethyl ester (fura-2/AM), glycine, ionomycin, joro spider toxin, *N*-methyl-D-aspartic acid (NMDA), nifedipine, poly-L-lysine, 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo[*f*]quinoxaline-7-sulfonamide (NBQX), tetrodotoxin acetate (TTX) (Sigma-Aldrich, Deisenhofen, Germany); ω-conotoxin MVIIC, kainic acid (Tocris Cookson, Bristol, UK); horse anti-mouse immunoglobulin G, avidin–biotin complex (ABC Elite Kit), normal horse serum (Vector Laboratories, Burlingame, CA, USA). All other chemicals for the buffer and superfusion solutions were obtained from Sigma-Aldrich.

Stock solutions (10–30 mM) were prepared with distilled water or in DMSO (cyclopiazonic acid, cyclothiazide, diazoxide, GYKI 53655, NBQX, nifedipine) and appropriately diluted with superfusion medium. The final concentration of DMSO did not exceed 0.1% and had no effect on $[Ca^{2+}]_i$ when given alone.

2.2. Cortical cell cultures

Neuronal cultures were prepared from the cerebral cortex of 16-day-old rat embryos (E16; own strain WIST/Lei) as previously described (Hansson and Rönnbäck, 1989; Scheibler et al., 1999). Briefly, minced cortical tissue was dissociated by trypsinization [5 ml 0.25% trypsin in Gibco solution plus 5 ml Hanks'-balanced salt solution without Ca^{2+} and Mg^{2+}, and 100 μl (≥2000 U) DNase] at 37 °C for 4 min and careful trituration through a fire-polished Pasteur pipette in the same solution containing 1 ml culture medium (Dulbecco's modified Eagle medium/Nutrient mixture F12; supplemented with 20% foetal heat-inactivated bovine serum, 2.5 mM L-glutamine, 36 mM D(+)-glucose, 15 mM HEPES, 50 μg/ml gentamycin, pH 7.4 adjusted with NaOH). Subsequently, the cell suspension was centrifuged at 1000 rpm for 10 min and the pellets were dissolved in fresh culture medium. Finally, isolated neurones (30 μl aliquots with 150.000 cells) were seeded in the centre of glass coverslips precoated with poly-L-lysine and cultured in 35-mm dishes with 2 ml culture medium. The cells were maintained at 37 °C in a humidified atmosphere of 95% air/5% CO_2 and were used for experiments after 10 to 15 days in vitro. Proliferation of nonneuronal cells was prevented by the addition of cytosine-β-D-arabinofuranoside (10 μM) for 24 h after 6 days in vitro.

2.3. GABA immunocytochemistry

Cell cultures were briefly washed with phosphate buffered saline (PBS, 0.1 M, pH 7.4), fixed for 20 min in 4% paraformaldehyde/0.25% glutaraldehyde at 4 °C, washed two times with Tris buffered saline (TBS, 0.05 M, pH 7.4) and then treated for 25 min with 1% H_2O_2 to inactivate endogenous peroxidase. After intensive washing with TBS and preincubation for 30 min with a 'blocking solution' (10% normal horse serum and 0.1% Triton X-100 in TBS, room temperature), the cultures were exposed overnight to the primary antibody (mouse monoclonal anti-GABA antibody, clone: 5A9; 1:1000) at 4 °C. Incubation with biotinylated horse anti-mouse immunoglobulin G (H+L; 1:65) for 1 h at room temperature and ABC Elite Kit (1:50, Vectastain) for 1 h was followed by treatment with 3,3′ -diaminobenzidine containing 1% $(NH_4)_2Ni(SO_4)_2$ plus 1% $CoCl_2$ (DAB–Ni/Co) and H_2O_2 to visualize the labelled cells. The stained cultures were dehydrated by increasing concentrations of ethanol, processed through *n*-butylacetate and coverslipped

with entellan. Control experiments were performed without the primary antibody.

2.4. AMPA-induced Co^{2+} uptake

Co^{2+} labelling was performed as previously described (Pruss et al., 1991) with minor modifications. This technique permits the identification of cells expressing functional AMPA/kainate receptors that are permeable to divalent cations. Cell cultures were incubated for 10 min in an 'uptake buffer' (composition in mM: sucrose 139, NaCl 57.5, KCl 4.8, $MgCl_2$ 2, $CaCl_2$ 1.3, D(+)-glucose 10, HEPES 10, pH 7.4) at room temperature. Subsequently, cultures were exposed to 5 mM $CoCl_2$ and 100 μM AMPA (plus cyclothiazide 50 μM to block AMPA receptor desensitization; D-AP5 10 μM was included in all experiments to inhibit NMDA channels) in 'uptake buffer' for 10 min at room temperature. In control experiments, cultures were preincubated with the AMPA receptor antagonist NBQX 30 μM 10 min before and during the 10 min exposure to the AMPA/$CoCl_2$ solution. In some experiments, high K^+ (30 mM; equimolar substitution of KCl for NaCl) or cyclothiazide (50 μM) without AMPA were tested. Then, the cultures were washed in 'uptake buffer' alone and subsequently for 5 min in 'uptake buffer' plus 2 mM EDTA to remove extracellular Co^{2+}, followed by 2 min incubation in $(NH_4)_2S$ (0.1%) to precipitate intracellular Co^{2+}. After three washes in fresh 'uptake buffer' (5 min), the cultures were fixed for 30 min in 4% paraformaldehyde (in 0.1 M PBS). For silver intensification, the cultures were washed three times in 'development buffer' (composition in mM: sucrose 292, hydroquinone 15.5, citric acid 42) and incubated for 30–45 min in 'development buffer' plus 0.1% $AgNO_3$ at 50 °C. The solution was changed at 15-min intervals and the reaction (visualization of brown coloured cells) was stopped by washing in warm 'development buffer'. After cooling, the staining was further intensified and stabilized by incubation in 1 mM AuCl (1–2 min), 2% CH_3CO_2Na (1 min), 2% $Na_2S_2O_3$ (2 min) and another washing in 2% CH_3CO_2Na (all at 4 °C; see Launey et al., 1998). The stained cultures were dehydrated in a series of graded ethanol and toluol and finally coverslipped with entellan (see above).

2.5. Fura-2 microfluorimetry

Cortical neurones were loaded with the Ca^{2+}-sensitive fluorescent dye, fura-2/AM (5 μM) at 37 °C for 30 min in culture medium. Then, the cells were incubated for an additional 30 min in a fura-2-free physiological saline (composition in mM: NaCl 133, KCl 4.8, KH_2PO_4 1.2, $MgCl_2$ 1, $CaCl_2$ 1.3, HEPES 10, D(+)-glucose 10; pH 7.4 adjusted with NaOH) at room temperature to remove extracellular traces of the dye and to complete de-esterification. Subsequently, the coverslips were mounted cell-side up in the free bottom of a perfusion chamber (250 μl), placed on the stage of an inverted microscope with epifluorescence optics (Diaphot 200, Nikon, Japan). Throughout the experiments, cells were continuously superfused (at 0.8 ml/min) by

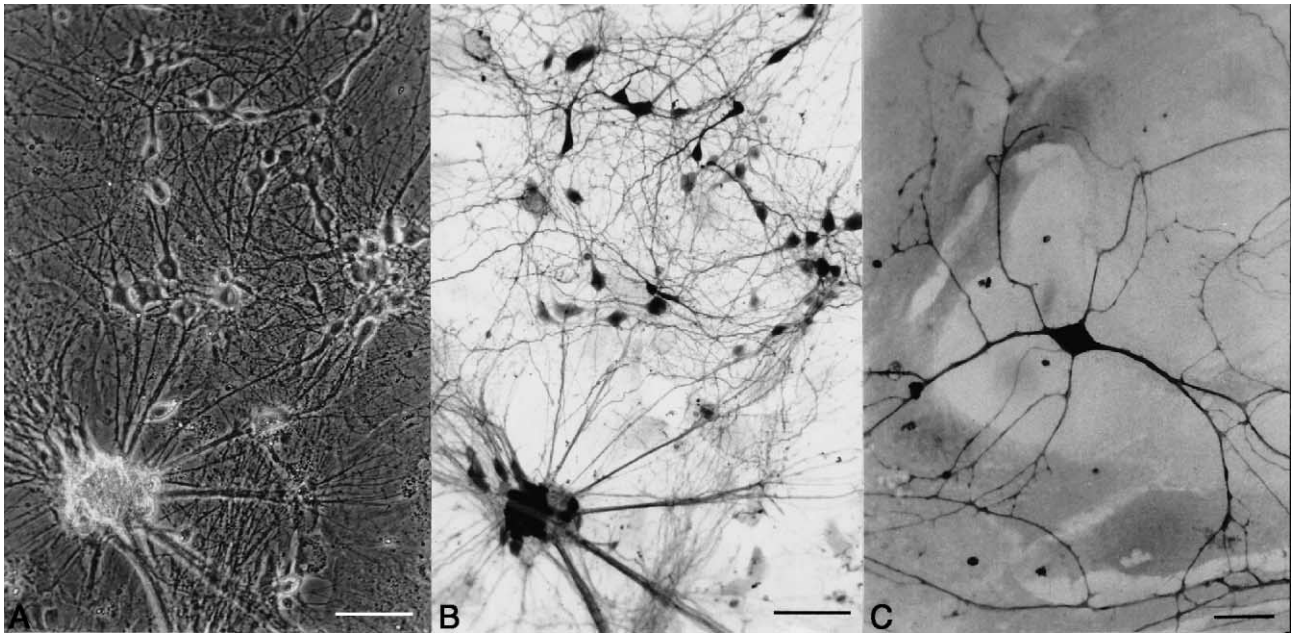

Fig. 1. Primary cultures of rat cortical neurones (E16, 14 days in vitro). (A) Phase-contrast photomicrograph. Representative field with a characteristic network of neurones. Some neurones form cell aggregates (see bottom left) that may be interconnected with each other by relatively thick bundles of neuronal processes. (B) GABA immunostaining (same field as shown in A). Note that the majority of identified neurones display GABA-positive immunoreactivity. Scale bars in A and B 200 μm. (C) Morphology of a characteristic, multipolar medium-sized GABA-immunopositive neurone. The presence of GABA in the glial cells (forming part of the glial bed in the culture) causes a moderate background staining. Scale bar 50 μm.

means of a roller pump with drug-free or drug-containing solutions, respectively. Different superfusion solutions were selected with a valve bank coupled to several reservoirs. Fluorescence ratio measurements were performed at room temperature on morphologically identified nonpyramidal neurones (multipolar, medium-sized) with a dual-wavelength spectrometer. Fura-2 fluorescence (over the cell somata), excited alternatively at 340 and 380 nm, was measured at 510/520 nm by a microscope photometer attached to a photomultiplier detection system (Ratiomaster System; PTI). Complete data acquisition, presentation and analysis were performed computer-controlled by using commercially available software (FeliX, Version 1.1; PTI). Calibration of $[Ca^{2+}]_i$ was performed according to Grynkiewicz et al. (1985): Ca^{2+}-saturated fura-2 signals (R_{max}) were determined in the presence of 10 μM ionomycin (Mg^{2+}-free buffer), and Ca^{2+}-free signals (R_{min}) with 25 mM EGTA (Ca^{2+}-free buffer). In order to generate concentration–response curves, AMPA (0.3–300 μM) or NMDA (1–300 μM) (plus 10 μM glycine, Mg^{2+}-free solution) were applied at up to four different concentrations to the same neurone in random order, for 60 s and with an interval of 10 min (cells not responding up to 30 or 100 μM AMPA or NMDA were excluded from the calculation). The AMPA (or NMDA)-induced rise of $[Ca^{2+}]_i$ was defined as the peak increase in the Δ fluorescence ratio (i.e. the fluorescence ratio 340/380 nm in response to AMPA minus the basal fluorescence ratio). The data were calculated as means ± S.E.M. of *n* determinations per tested concentration and the curves were fitted by a four-parameter logistic function (SigmaPlot, Version 5.0; SPSS), which gives the EC_{50} values (concentrations causing 50% of the maximum effect) and the slope of the curves. A submaximal concentration of AMPA (30 μM) was chosen for further experiments. To investigate the variability of the cell signals, the response to AMPA (30 μM) was tested in a total of 501 neurones. The viability of nonreactive neurones was verified by stimulations with 30 mM K^+ (equimolar substitution of KCl for NaCl). In most other studies, AMPA (30 μM) was applied three times (S_1, S_2, S_3) for 60 s every 10 min. A Ca^{2+}-free medium (plus 1 mM EGTA), cyclopiazonic acid, tetrodotoxin, nifedipine, ω-conotoxin MVIIC, calcicludine, flunarizine, NBQX, CNQX, GYKI 53655, CPP, R-AP5 and joro spider toxin were superfused 10 min before and during S_2. Cyclothiazide and diazoxide were

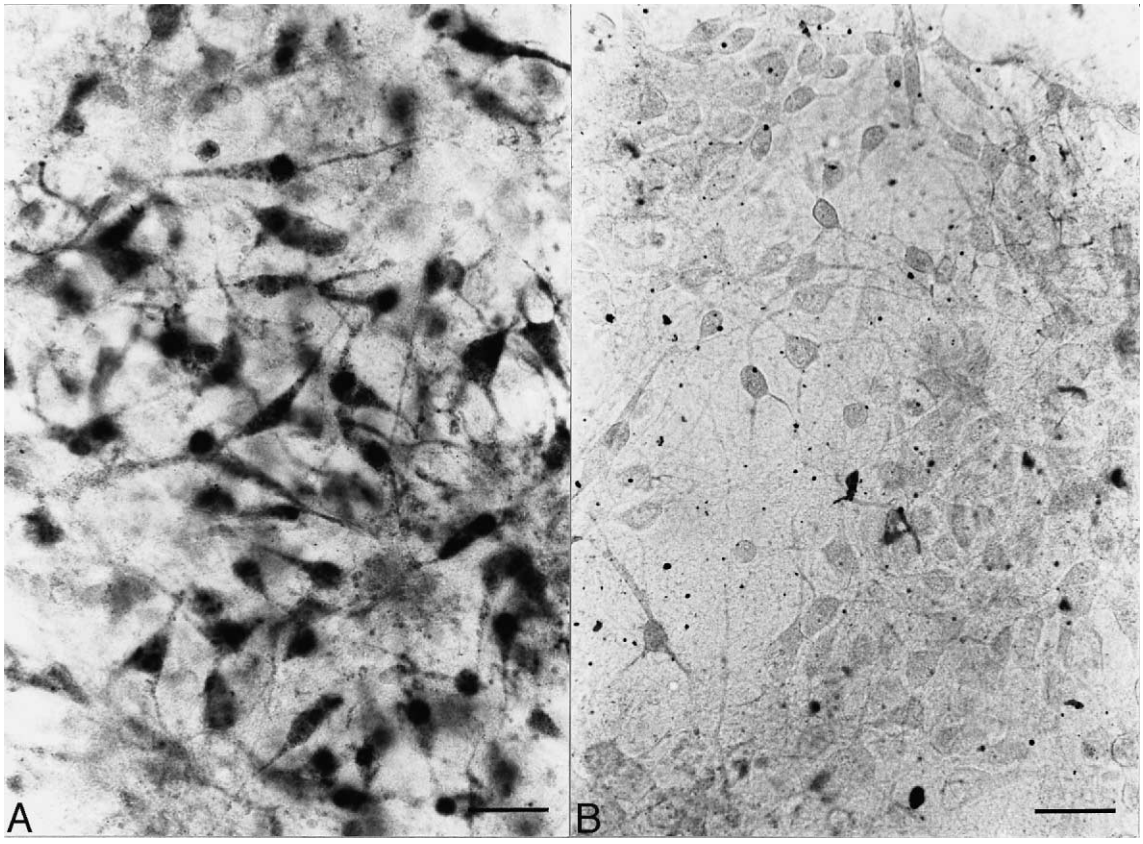

Fig. 2. AMPA-induced Co^{2+} uptake labelling of cultured cortical neurones. (A) Cell cultures were exposed to 100 μM AMPA, 50 μM cyclothiazide, 10 μM R-AP5 and 5 mM $CoCl_2$ (Co^{2+} precipitation, hot silver-development and gold-intensification as described in Materials and methods). Many neurones stain positively. Note the punctate labelling on somata and proximal dendrites; nuclear darkening is also prominent. (B) Sister cultures were treated as described in A, but additionally preincubated with the AMPA receptor antagonist NBQX (30 μM) 10 min before and during the AMPA/$CoCl_2$ exposure. In contrast to A, no cellular labelling could be observed. Scale bars 100 μm.

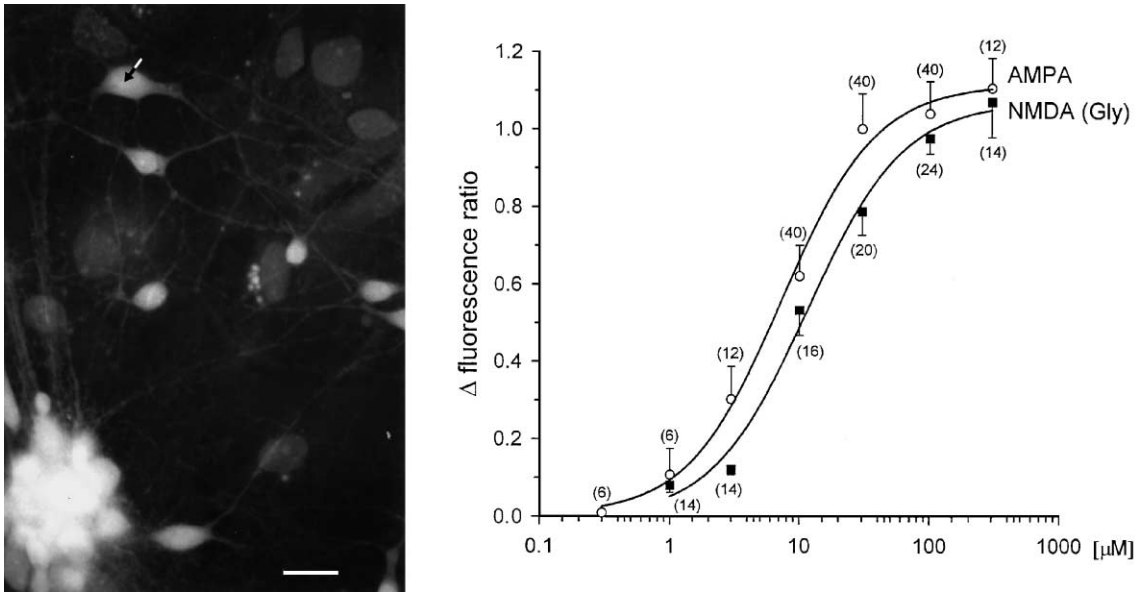

Fig. 3. Concentration–response curves for the AMPA- and NMDA-induced increase in $[Ca^{2+}]_i$ in cultured nonpyramidal cortical neurones. Δ Fluorescence ratios were calculated from the difference between the peak response to each AMPA or NMDA (plus 10 μM glycine, Mg^{2+}-free solution) concentration and the corresponding basal value. All data are expressed as means ± S.E.M. of *n* determinations per concentration (number of cells in parentheses). The sigmoidal curves were fitted using a four-parameter logistic function (SigmaPlot). The calculated EC_{50} values were 7.35 ± 1.18 μM (slope factor 1.21 ± 0.23) and 11.56 ± 2.92 μM (1.19 ± 0.38) for the AMPA- and NMDA-curve, respectively. In the photomicrograph on the left, the arrow indicates a multipolar nonpyramidal neurone as used in the experiments (fura-2 fluorescence after fixation and embedding). Scale bar 50 μm.

superfused together with AMPA (during S_2). Drug effects were evaluated as the percentage change of the AMPA-induced signal at S_2 versus the signal at S_1. All data are expressed as means ± S.E.M. of *n* determinations as a percentage of the AMPA control response at S_1. The difference between the AMPA-induced signals at S_1 and S_2 (absolute values) was assessed for statistical significance using the parametric paired Student's *t*-test after passing the normality test (SigmaStat, Version 2.0; SPSS). In the absence of additional drugs, the AMPA (30 μM)-induced rise in $[Ca^{2+}]_i$ was reproducible (1.9 ± 0.5% decrease from S_1 to S_2, $n = 10$ cells; $P > 0.05$; see Fig. 5). Cells exhibiting excessive rundown of the AMPA response were omitted from analysis. In some cases, NMDA or kainic acid were used as agonists in a similar manner as described for AMPA. The increase by NMDA (100 μM) or kainate (100 μM) of $[Ca^{2+}]_i$ was also reproducible on two consecutive applications (not shown).

3. Results

3.1. GABA immunocytochemistry

Immunocytochemical studies revealed that approximately 80–90% of neurones in the cortical cell cultures used were GABA-positive. The majority of the neuronal cell population, therefore, seemed to represent nonpyramidal interneurone-like cells. However, in 10- to 15-day-old cultures, the neurones form cell aggregates and the exact cell number is difficult to estimate. Fig. 1 demonstrates by a direct comparison of the same field in the culture dish that many neurones (Fig. 1A: phase-contrast photomicrograph) could be identified by GABA immunostaining (Fig. 1B). Fig. 1C shows a characteristic multipolar, medium-sized GABA-immunoreactive neurone. The dendrites exhibit an irregular branching pattern, thickened branching points,

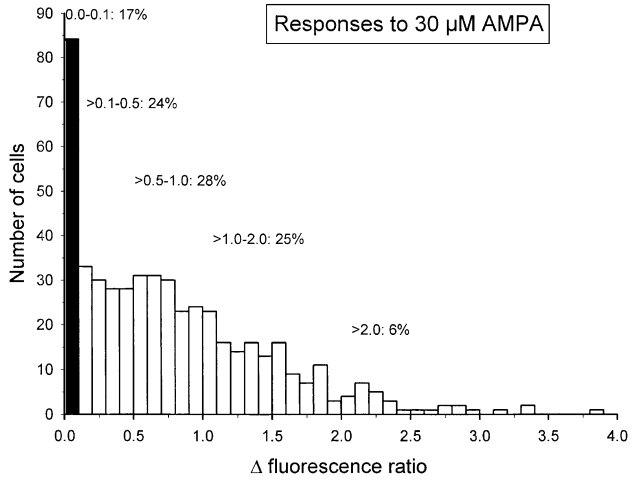

Fig. 4. Distribution of AMPA response amplitudes (Δ fluorescence ratios) from 501 nonpyramidal cortical neurones stimulated with 30 μM AMPA (always first response).

numerous varicosities and thorn-like excrescences. An axon cannot clearly be recognized. This cell type could be frequently observed and was selected for the fura-2 experiments. The morphology of single GABAergic neurones, however, showed a large variability regarding the form of the somata as well as the dendritic arborization (small cells with ovoid shape, fusiform or large multipolar cells could be also observed), possibly indicating special subpopulations of interneurone-like cells.

3.2. AMPA-stimulated Co^{2+} uptake

A combined pharmacological–histological method, the AMPA-stimulated Co^{2+} staining technique completed with a specific silver–gold impregnation procedure was used to label cortical neurones bearing Ca^{2+}-permeable AMPA receptors. After stimulation with AMPA (100 μM; alone), Co^{2+} accumulation was detected sparsely in about 5% of the neurones. Co-application of cyclothiazide (50 μM), a desensitization blocker of AMPA receptors, considerably increased the number of stained cells to more than 50% (Fig. 2A). In contrast, in identical cultures after exposure to the selective AMPA receptor antagonist NBQX (30 μM), no labelling was observed (Fig. 2B). Stimulation with high K^+ (30 mM) or cyclothiazide (50 μM) without AMPA was not able to induce Co^{2+} staining.

3.3. Fura-2 microfluorimetry

As mentioned above, single nonpyramidal medium-sized neurones with four to six dendrites were selected for these experiments and fluorescence ratio measurements were made over the cell somata (see photomicrograph in Fig. 3). AMPA (0.3–300 μM) caused a concentration-dependent increase of $[Ca^{2+}]_i$ in about 80% of the investigated neurones. Fig. 3 shows the corresponding concentration–response curve. A maximal response was estimated by applying 300 μM AMPA and the EC_{50} value was 7.35 ± 1.18 μM. Interestingly, the potency of AMPA was comparable with that of NMDA (plus 10 μM glycine, Mg^{2+}-free solution; EC_{50} value 11.56 ± 2.92 μM).

A submaximal concentration (30 μM) of AMPA was employed in all subsequent experiments. This concentration induced a mean increase of the Δ fluorescence ratio by 1.02 ± 0.09, which corresponds to an increase of $[Ca^{2+}]_i$ from basal 85.6 ± 6.7 to 491.2 ± 23.3 nM ($n = 10$ cells). However, marked cell-to-cell variability in the magnitude of the $[Ca^{2+}]_i$ response could be observed. The sequential histogram (Fig. 4) illustrates the distribution of Δ fluorescence ratios from 501 neurones tested during all the experiments. About 17% of the cells showed no clear increase in $[Ca^{2+}]_i$, 24% exhibited a weak signal, 53% had Δ fluorescence ratios of 0.5 to 2.0 and about 6% showed larger amplitudes up to ratios of 4.0.

In order to investigate the influence of various pharmacological treatments on the AMPA-induced increase in $[Ca^{2+}]_i$, AMPA (30 μM, for 60 s) was applied three times every 10 min in the following studies. In viable cell cultures, AMPA induced an immediate increase in $[Ca^{2+}]_i$ and after removal of the agonist, the level of $[Ca^{2+}]_i$ returned to the prestimulus baseline level within 3–5 min of superfusion with drug-free physiological solution. The amplitudes were reproducible after a 10-min washout interval (representative traces are shown in Fig. 5A). At high amplitudes of Ca^{2+} signals (fluorescence ratios >2), the AMPA response sometimes persisted longer and the amplitudes decreased with the second and third stimulation, possibly indicating first signs

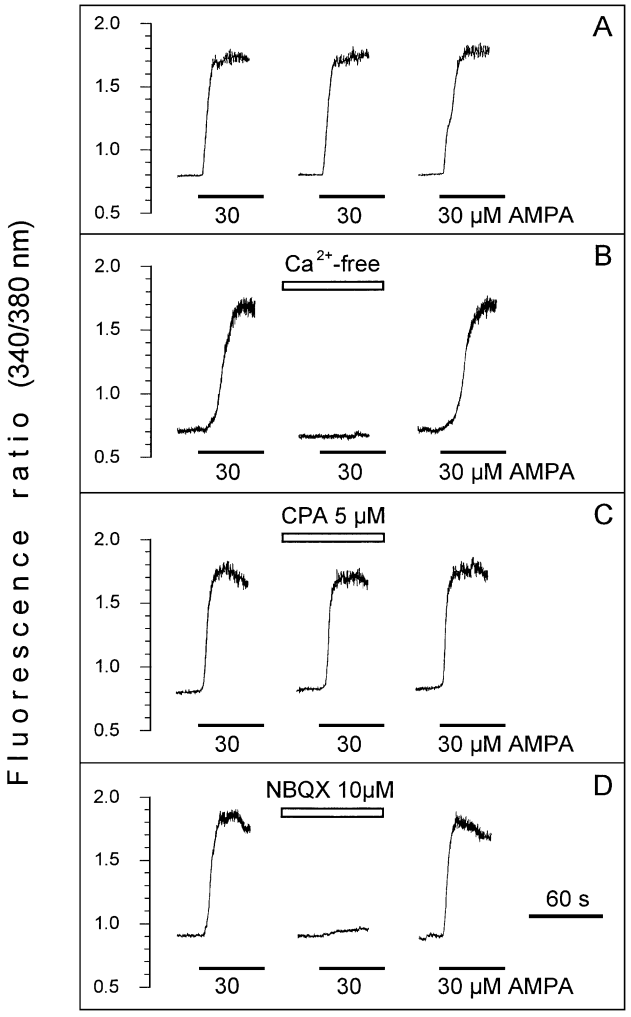

Fig. 5. Intracellular Ca^{2+} responses elicited by AMPA stimulation in single multipolar nonpyramidal neurones, based on fluorescence ratio measurements. The cultures were superfused with physiological solution and stimulated three times with AMPA (30 μM) for 60 s every 10 min (as indicated by the horizontal bars). All other drugs were superfused 10 min before and during the second stimulation period. Representative tracings from single experiments are documented. (A) Stimulation with AMPA (30 μM) three times, illustrating reproducible response amplitudes (control experiment). (B) Dependence of AMPA response on extracellular Ca^{2+} (Ca^{2+}-free solution plus 1 mM EGTA). (C) Effect of intracellular Ca^{2+} depletion by the endoplasmatic Ca^{2+}-ATPase inhibitor cyclopiazonic acid (5 μM). (D) Inhibition of AMPA response by the competitive AMPA receptor antagonist NBQX (10 μM).

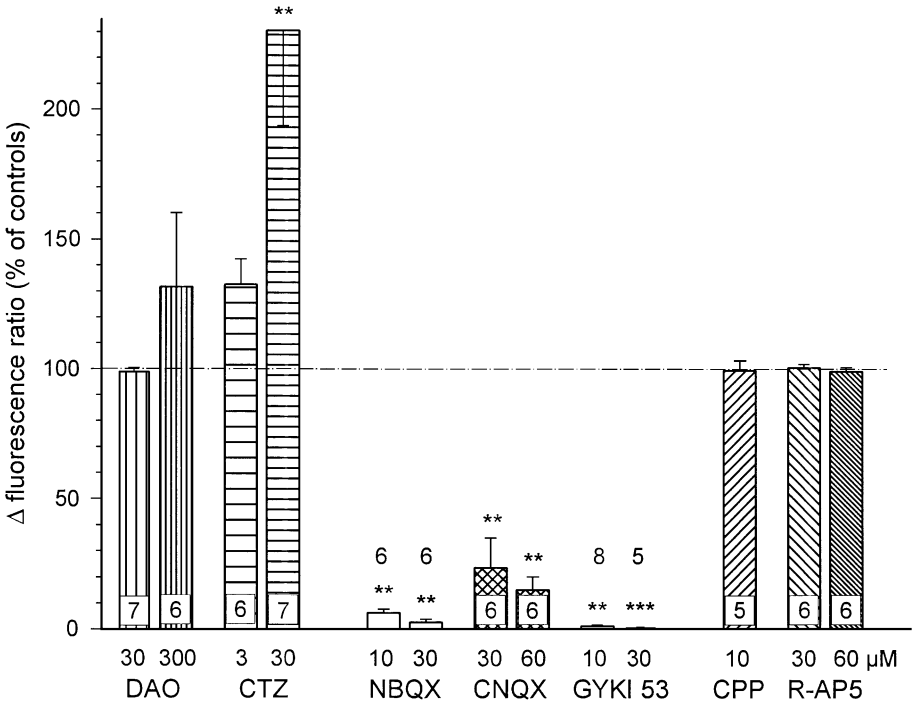

Fig. 6. Effect of the positive allosteric modulators diazoxide (DAO) and cyclothiazide (CTZ) as well as various AMPA (NBQX, CNQX, GYKI 53655) and NMDA receptor antagonists (CPP, R-AP5) on the AMPA (30 μM)-induced increase of $[Ca^{2+}]_i$ in cultured cortical nonpyramidal neurones. AMPA (30 μM) was superfused three times for 60 s every 10 min (S_1-S_3). Whereas diazoxide and cyclothiazide were co-applied with AMPA at S_2, the AMPA or NMDA receptor antagonists were applied 10 min before and during the second AMPA application. The concentrations of all drugs are indicated. Data are shown as means ± S.E.M. and are expressed as a percentage of the control response at S_1. Numbers of cells tested are indicated at the base of each column. $**P<0.01$, $***P<0.001$.

of cellular damage. In a Ca^{2+}-free medium (plus 1 mM EGTA), the response to AMPA was almost abolished (99.3 ± 0.3% inhibition; $n=6$; $P<0.001$; see Fig. 5B), but in normal Ca^{2+} medium, the response was not altered after depletion of the intracellular Ca^{2+} stores by cyclopiazonic acid (5 μM) (0.8 ± 3.3% inhibition; $n=5$; $P>0.05$; see Fig. 5C). The AMPA-induced Ca^{2+} influx was insensitive to the Na^+ channel blocker tetrodotoxin (0.3 μM) (0.5 ± 3.9% inhibition; $n=6$; $P>0.05$) and various Ca^{2+} channel blockers such as nifedipine (10 μM) (16.8 ± 6.6%; $n=7$; $P>0.05$), ω-conotoxin MVIIC (0.1 μM) (7.2 ± 5.1%; $n=7$; $P>0.05$) or flunarizine (1 μM) (5.3 ± 4.8%; $n=8$; $P>0.05$). Only calcicludine (0.1 μM) caused a small, but significant inhibitory effect (17.9 ± 4.8%; $n=8$; $P<0.05$). Some additional experiments with Cd^{2+} as an inorganic Ca^{2+} channel blocker showed at 50 μM (superfusion 5 min, no increase in the basal fluorescence ratio); no significant decrease of the AMPA response (11.7 ± 7.5%; $n=7$, $P>0.05$), whereas the response to high K^+ (30 mM) was markedly inhibited (68.7 ± 4.4%; $n=6$; $P<0.05$). Higher concentrations of Cd^{2+} (100–1000 μM) interfered with the fura-2 fluorophore and caused a slow and irreversible increase in the basal fluorescence ratio. On the other hand, positive allosteric modulators of AMPA receptors such as diazoxide (300 μM) and cyclothiazide (30 μM) tended to increase, or increased, the AMPA-induced Ca^{2+} influx (Fig. 6). It is noteworthy that cyclothiazide alone induced a transient increase in $[Ca^{2+}]_i$ and marked Ca^{2+} oscillations at higher concentrations (≥ 10 μM). The selective AMPA receptor antagonists NBQX and GYKI 53655 (10 and 30 μM; Figs. 5D and 6), completely blocked the AMPA response. The AMPA/kainate receptor antagonist CNQX (30 and 60 μM) also inhibited the increase of $[Ca^{2+}]_i$ by AMPA, whereas the selective NMDA receptor antagonists CPP (10 μM) and R-AP5 (30 and 60 μM) caused no change (Fig. 6). Some additional experiments with a polyamine blocker of AMPA receptor channels, joro spider toxin (0.5 μM), indicated striking differences in efficacy between single cells, varying from no effect to 65% inhibition (mean 24.6 ± 6.3%, $n=12$ cells; $P<0.05$).

4. Discussion

Primary cultures of neurones are widely used in pharmacological and electrophysiological studies and it is often essential to know which cell types are present for an accurate interpretation of the results (Janssens and Lesage, 2001). Our morphological investigations documented that the large majority (80–90%) of the cultured rat cortical neurones was immunoreactive for GABA. Some preliminary studies revealed that numerous GABA-positive neurones displayed also parvalbumin immunoreactivity, some neurones exhibited calretinin or rarely calbindin immuno-

reactivity (Franke and Fischer, unpublished results), indicating different families of inhibitory cell types (Kawaguchi and Kubota, 1997). These results show that under the described culture conditions, a disproportionately high number of interneurone-like cells with undirected dendritic processes will survive and grow out (see Hertz et al., 1989). For the fura-2 experiments, we preferentially used medium-sized multipolar neurones (see Figs. 1C and 3).

The histological Co^{2+} staining technique, first described by Pruss et al. (1991), was shown to identify neurones expressing Ca^{2+}-permeable AMPA/kainate receptor channels in various cell and tissue preparations (Bardoul et al., 1998; Jensen et al., 1998; Launey et al., 1998). Our study indicates that a similar approach with AMPA/cyclothiazide stimulation visualizes intracellular Co^{2+} accumulation in the cortical cell cultures used. In agreement with studies of Jensen et al. (1998), stimulation with AMPA alone resulted in labelling of only a small number of cortical neurones. Cyclothiazide considerably enhanced the staining, indicating a high dependence on AMPA receptor desensitization. However, in partial contradiction to previous studies, which suggest that about 15% or 22% of neurones in cortical cultures express Ca^{2+}-permeable non-NMDA receptors (Turetsky et al., 1994; Jensen et al., 1998), our investigations using an additional gold-staining intensification procedure, revealed more than 50% of neurones with Co^{2+} labelling. König et al. (2001) demonstrated recently that the Ca^{2+} concentration in the cells remained fairly low after AMPA (50 μM) application until cyclothiazide (50 μM) was added. Obviously, a high and long-lasting Co^{2+} accumulation into a single neuron seems to be necessary for histological staining, which can explain the discrepancy that 80% of the neurones studied showing an AMPA-induced increase in $[Ca^{2+}]_i$, but only 5% become labelled with Co^{2+}. In agreement with data in the literature, the specificity of the Co^{2+} staining technique is underlined by the ability of NBQX to inhibit the AMPA-induced Co^{2+} uptake. Also, stimulation with high K^+ alone was not able to induce Co^{2+} labelling, reflecting the relative inability of Co^{2+} to permeate voltage-sensitive Ca^{2+} channels or NMDA channels (Jensen et al., 1998; Yin et al., 1999).

The fura-2 experiments show that the stimulation by the selective agonist (*S*)-AMPA induced a concentration-dependent increase in $[Ca^{2+}]_i$ in the majority of cells tested. The considerable cell-to-cell variability indicates that individual neurones express AMPA receptor channels with different Ca^{2+} permeabilities or a variable density of Ca^{2+}-permeable AMPA receptors. NMDA (plus 10 μM glycine) and AMPA caused a similar rise in $[Ca^{2+}]_i$ (see also Scheibler et al., 1999). Thus, in these neurones, the functional dichotomy between NMDA and AMPA receptors regarding the permeability to Ca^{2+} seems to be less marked than primarily discussed in the literature (Burnashev, 1996).

The (*S*)-AMPA-induced increase in $[Ca^{2+}]_i$ can be regarded as widely selective for AMPA receptors for at least five reasons. First, the response was dependent on the extracellular Ca^{2+} concentration and not on Ca^{2+} release from intracellular stores as shown in experiments with Ca^{2+}-free medium and cyclopiazonic acid, a specific inhibitor of endoplasmatic Ca^{2+}-ATPase. Second, the Ca^{2+} influx was mostly due to the entry of Ca^{2+} via the receptor channel itself. The opening of voltage-sensitive Na^+ or Ca^{2+} channels as a possible consequence of the AMPA-induced membrane depolarization (Carriedo et al., 1998) failed to considerably contribute to the observed rise of $[Ca^{2+}]_i$. Only the inhibition by calcicludine, a potent blocker of neuronal L-type Ca^{2+} channels (Stotz et al., 2000; for selectivity of various Ca^{2+} channel blockers, see Adachi-Akahane and Nagao, 2000), did reach significance level. Third, the AMPA response was virtually abolished by GYKI 53655, a potent and selective noncompetitive AMPA receptor antagonist (Michaelis, 1998; Tarnawa and Vizi, 1998) as well as by the competitive AMPA receptor antagonist NBQX, which has improved AMPA receptor selectivity compared to CNQX (Bräuner-Osborne et al., 2000). Fourth, the selective NMDA receptor antagonists R-AP5 and R-CPP (Bräuner-Osborne et al., 2000) did not alter the AMPA effect. Fifth, the positive allosteric modulator cyclothiazide, which selectively inhibits desensitization of AMPA receptors (Partin et al., 1993; Yamada, 1998), enhanced the Ca^{2+} signal in the majority of neurones when co-administered with AMPA (see also Ambrósio et al., 2000). The potency of diazoxide, another benzothiadiazine derivative (Yamada, 2000), was lower than that of cyclothiazide. Recently, it has been suggested that several positive allosteric modulators may act at multiple recognition sites (Kapus et al., 2000), which can explain different effects. Finally, experiments with joro spider toxin, which acts as an open channel inhibitor of Ca^{2+}-permeable AMPA receptors lacking the edited GluR2 subunit (Iino et al., 1996; Ozawa et al., 1998), exhibited variable but mostly small degrees of inhibition in the cells tested. A possible explanation could be the known voltage-dependence of the toxin block (Iino et al., 1996) and/or the presence of various levels of the GluR2 subunit in interneurones, documented recently in the literature (Washburn et al., 1997; He et al., 1998).[1] Moreover, individual neurones may express both Ca^{2+} permeable and Ca^{2+} impermeable AMPA receptors (Albuquerque et al., 1999; König et al., 2001).

Interestingly, functional AMPA receptors permeable to Ca^{2+} are already present at very early stages of embryonic development (König et al., 2001). The activation of such AMPA receptor channels in neuronal subpopulations, such as GABAergic interneurones, can trigger an increase in $[Ca^{2+}]_i$ important for intracellular signaling pathways including long-lasting changes in synaptic efficacy (Geiger et al., 1995; Perkinton et al., 1999; Ross and Soltesz, 2001). The AMPA-induced membrane depolarization can also increase the cell excitability, resulting in an enhancement

[1] Numerous nonpyramidal GABAergic neurones in the cultures studied seem to be immunopositive for the GluR2 subunit (Franke and Fischer, unpublished results).

of inhibitory drive to other postsynaptic neurones. Because single interneurones influence thousands of postsynaptic principal cells, they have a powerful regulatory role in neuronal circuits. On the other hand, compensatory mechanisms for Ca^{2+} buffering and Ca^{2+} extrusion (expression of Ca^{2+}-binding proteins like parvalbumin, effective Ca^{2+} membrane pumps) may contribute to the relative resistance of these neurones to various pathological conditions such as ischemia, physical trauma or epileptic seizures (Pellegrini-Giampietro et al., 1997; Weiss and Sensi, 2000).

The morphological and pharmacological results of the present study demonstrate that the majority of the cortical GABAergic interneurone-like cells studied possess Ca^{2+}-permeable AMPA receptors that could participate in a variable degree in Ca^{2+} influx signalling. Thus, given the large amplitudes of Ca^{2+} responses caused by AMPA in individual cells, it appears that the AMPA receptors involved have an important role in mediating intracellular and intercellular signal transduction in the cerebral cortex.

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